IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of
Masakazu SATO, et al.
Serial No. 10/518,788 Art Unit: 1623
Filed: August 8, 2003 Examiner: MCINTOSH III, TRAVISS C
For: ARYL 5-THIO-β-D-GLUCOPYRANOSIDE DERIVATIVES AND THERAPEUTIC AGENTS FOR DIABETES CONTAINING THE SAME

D E C L A R A T I O N

Commissioner for Patents
P.O.Box 1450
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Sir:

I, Masayuki Arai, a Japanese citizen, residing at 28, Kitamoto 2-Chome, Kitamoto-shi, Saitama, 364-0006, Japan, do hereby solemnly and sincerely declare that:

I graduated from Science University of Tokyo in March, 1982 with a B.S. degree; I went on to graduate school in the same university and graduated in March, 1994 with a master degree; My major was natural product chemistry; I isolated and characterized a compound which exterminate termite;

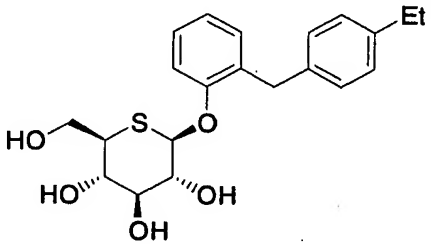
I began employment with Taisho Pharmaceutical Co., Ltd., the Assignee of the above-identified application, in April 1984; I have been belonged to Drug Metabolism Laboratory; I have been concerned with drug metabolism and pharmacokinetics ever since I joined the company;

My work, for example, is an evaluation of drug plasma

concentration and tissue distribution;

In order to evaluate an oral absorption of the claimed compound in the above-identified application, I conducted the following experiment.

A test compound is the compound of Example 24 (compound 1) of the specification of the above-identified application, represented by the following formula:



WO04/014931
example 24

"Oral Absorption Test"

(1) Analyte preparation for drug concentration measurement after intravenous administration

A test compound (323 mg) was dissolved in a 22.5 w/v% aqueous hydroxypropyl-β-cyclodextrin (HP-β-CD) solution to prepare a 2.90 mg/ml solution. Dogs (beagle, 6 months old, 7.62 to 8.85 kg, n=5) were each administered with the test compound at a dose of 3 mg/kg via the cephalic vein.

Then, an injection needle (22G) attached to a syringe (2.5 mL) was used to collect about 1.5 mL blood via the cephalic vein. The timing of blood collection was set to 2, 5, 10 and 30 minutes and 1, 2, 4, 8, 12 and 24 hours after administration. Each blood sample was transferred to a test tube containing heparin sodium, mixed well and cooled on ice, followed by centrifugation (settings: 3,000 rpm, 10 minutes, 4°C) to obtain about 0.5 mL plasma.

(2) Analyte preparation for drug concentration measurement after oral administration

A test compound (243 mg) was dissolved in a 22.5 w/v% aqueous HP-β-CD solution to prepare a 1.49 mg/ml solution. Dogs (beagle, 6 months old, 7.62 to 8.85 kg, n=5) were each administered intragastrically with the test compound at a dose of 3 mg/kg by forced oral administration using an oral catheter attached to a syringe (20 mL). After administration, about 10 mL injectable water was further administered to wash out the solution remaining in the oral catheter. The timing of blood collection was set to 15 and 30 minutes and 1, 2, 4, 8, 12 and 24 hours after administration. Each blood sample was transferred to a test tube containing heparin sodium, mixed well and cooled on ice, followed by centrifugation (settings: 3,000 rpm, 10 minutes, 4°C) to obtain about 0.5 mL plasma.

(3) Drug concentration measurement

The compound concentration in plasma was determined in a manner shown in the following flow chart.

Blank sample, zero sample, standard sample for calibration curve, test sample (plasma volume: 25 μL for each sample)

- 50 μL internal standard solution (10 ng/mL)
  (50 μL water/methanol (1:1, v/v) for blank sample)
- 500 μL 100 mmol/L ammonium acetate solution

Empore C18 SD High Performance Extraction Disk Cartridge (4 mm/1 mL) (activated with 500 μL methanol and 500 μL 100 mmol/L ammonium acetate solution)

- 500 μL 100 mmol/L ammonium acetate solution (wash)
- 500 μL water/methanol (8:2, v/v) (wash)
- 500 μL water/methanol (2:8, v/v) (elute)

(Centrifugation; settings: 4°C, 2000 rpm, 1 minute)

Evaporation to dryness with nitrogen gas at 40°C (setting) under heated conditions

100 μL 0.1 vol% acetic acid solution/0.1 vol% acetic acid-containing methanol solution (7:3, v/v)

Ultrafree-MC (centrifugal filtration; settings: 4°C, 10000 rpm, 1 minute)

Injection of 15 μL into LC/MS/MS system*

* LC/MS/MS: API 3000 system, MDS Sciex

HPLC column: YMC-Pack Pro C18 (35 mm × 2.0 mm I.D., particle size: 3 μm), YMC

(3) Calculation of bioavailability (BA)

The compound concentrations obtained at individual time points during the above LC/MS/MS analysis were used to calculate bioavailability according to the following equation.

$$BA\ (\%) = \frac{(AUC_{0-\infty}\ \text{for oral administration})/(\text{dosage for oral administration})}{(AUC_{0-\infty}\ \text{for i.v. administration})/(\text{dosage for i.v. administration})} \times 100$$

(4) Results

The bioavailability of the compound of Example 24 (Compound 1) was 61.5%.

The undersigned declarant declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed this 3th day of October, 2007.

Masayuki Arai

Masayuki Arai

FEBS 20863

FEBS Letters 436 (1998) 71–75

Deglycosylation of flavonoid and isoflavonoid glycosides by human small intestine and liver β-glucosidase activity

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Abstract Flavonoid and isoflavonoid glycosides are common dietary phenolics which may be absorbed from the small intestine of humans. The ability of cell-free extracts from human small intestine and liver to deglycosylate various (iso)flavonoid glycosides was investigated. Quercetin 4′-glucoside, naringenin 7-glucoside, apigenin 7-glucoside, genistein 7-glucoside and daidzein 7-glucoside were rapidly deglycosylated by both tissue extracts, whereas quercetin 3,4′-diglucoside, quercetin 3-glucoside, kaempferol 3-glucoside, quercetin 3-rhamnoglucoside and naringenin 7-rhamnoglucoside remained unchanged. The K_m for hydrolysis of quercetin 4′-glucoside and genistein 7-glucoside was ~32±12 and ~14±3 μM in both tissues respectively. The enzymatic activity of the cell-free extracts exhibits similar properties to the cytosolic broad-specificity β-glucosidase previously described in mammals.

Key words: Quercetin 4′-glucoside; Genistein 7-glucoside; β-Glucosidase; Flavonoid; Flavonol; Isoflavonoid (human liver)

1. Introduction

Flavonoids are polyphenolic compounds found abundantly in plants, which may play a dietary role in reducing the risk from chronic diseases such as cardiovascular disease and cancer [1–3]. The flavonoids exist in nature almost exclusively as β-glycosides. The flavonols are found mainly as the 3-*O*-glycoside, although the 7 and 4′ positions may also be glycosylated in some plants, e.g. onions [4]. Other classes of flavonoids, such as the flavones, flavanones and isoflavones, are found mainly glycosylated in the 7 position [5]. The basic structure of the (iso)flavonoids with positions of glycosylation are shown in Fig. 1.

Flavonoid glycosides are mostly unmodified by various cooking methods [6], although the aglycone is released by fermentation and processes involving autolysis [7]. Thus, in general, food when consumed has a low level of aglycone compared to the glycosides. Until recently it has been assumed that the glycosides cannot be absorbed from the small intestine and cleavage of the β-glycoside linkage will not occur until the compounds reach the microflora in the large intestine [8]. Some evidence now exists for the preferential uptake of onion flavonol glycosides from the small intestine compared to the aglycone [9,10]. In vitro uptake of genistein 7-glucoside across Caco-2 cells in culture has also been shown [11]. There is little evidence to suggest how these flavonoid glycosides would be metabolized by tissues, as most human studies on the absorption and metabolism of flavonoids from foods have involved hydrolysis of the samples to the aglycones prior to analysis.

Biological activity depends on the presence or absence of the glycoside residue [12]. The position and nature of the sugar residue may increase the uptake of the compound in the small intestine. However, the aglycone is likely to have a greater biological effect than the glycoside [12], so deglycosylation via a β-glucosidase activity would be an important step in metabolism.

Three native β-glucosidase enzymes have been identified in humans [13]. Two of these, the glucocerebrosidase (EC 3.2.1.62) and lactase phlorizin hydrolase (EC 3.2.1.108), are membrane-bound and have been shown to have specific substrate activities. The former is a lysosomal enzyme which hydrolyses glucoceramide from endogenous membrane glycolipids; deficiency in this enzyme is associated with Gaucher disease [13]. Lactase phlorizin hydrolase is a membrane-bound enzyme found in the brush-border of the small intestine, and is primarily responsible for hydrolysis of lactose; deficiency of this enzyme causes lactose-intolerance [14]. Lactase phlorizin hydrolase also has a second hydrophobic domain which is capable of hydrolyzing phlorizin to phloretin and glucose [15]. The third β-glucosidase is a broad-specificity cytosolic enzyme found in abundance in the liver, kidney and small intestine of mammals [16,17].

The broad-specificity β-glucosidase is thought to play a role in detoxifying xenobiotics by hydrolyzing the β-glucoside moiety to provide a site for conjugation, which would lead to rapid excretion in the bile and urine. Several naturally occurring compounds have been shown to be substrates for this broad-specificity enzyme [18,19]. A high specificity was also shown towards β-glucose residues on steroids such as estradiol by rabbit β-glucosidase [16,20]. Pyridoxine 5′-β-glucoside is hydrolyzed to pyridoxine, which is the major active form of vitamin B_6, by a cytosolic β-glucosidase present in the small intestine, liver and kidneys of humans [21]. Recently another cytosolic β-glucosidase has been identified in porcine jejunal mucosa [22] which is specific for pyridoxine glucoside and may be responsible for the activity found previously in human tissue.

The aims of this study are to use human small intestine and liver cell-free extracts to show whether there is β-glucosidase activity towards (iso)flavonoid glycosides. By using known inhibitors, we show that the high activity present in human small intestine and liver cell-free extracts is likely to be attributed to the cytosolic broad-specificity β-glucosidase.

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2. Materials and methods

2.1. Chemicals

All reagents were purchased from Sigma (Poole, Dorset, UK) and were of analytical reagent grade or HPLC grade where applicable. Quercetin 4′-glucoside (Q 4′-glu) and quercetin 3,4′-diglucoside (Q 3,4′-glu) were extracted and purified (>99% purity) from onion bulb tissue [23]. Quercetin 3-glucoside (Q 3-glu), kaempferol 3-glucoside (K 3-glu), daidzein 7-glucoside (daidzin), apigenin 7-glucoside and naringenin 7-glucoside were purchased from Apin Chemicals (Abingdon, Oxon, UK). Quercetin 3-rhamnoglucoside (rutin), naringenin 7-rhamnoglucoside (naringin), and genistein 7-glucoside (genistin) were obtained from Sigma. All (iso)flavonoids were checked for purity by HPLC prior to use. Water was purified via a Millex Q-plus system (Millipore, Watford, UK).

2.2. Preparation of flavonoids

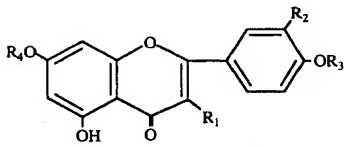
All flavonol glycosides were dissolved in methanol and stored at 4°C. Immediately prior to the reaction, an aliquot was dried by rotary evaporation and redissolved in dimethyl sulphoxide (DMSO) and potassium phosphate buffer (10 mM, pH 7), to give a final DMSO concentration of less than 0.2% (v/v) in the reaction mixture. Flavones, flavanones and isoflavones were dissolved in DMSO and stored at 4°C. As above, an aliquot was diluted in phosphate buffer to give a final DMSO concentration of less than 0.2% (v/v) in the reaction mixture. Standard curves were calculated for each (iso)flavonoid glycoside and corresponding aglycone.

2.3. Preparation of cell-free extracts

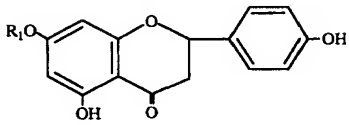
Samples of small intestine and liver were obtained from redundant tissue of surgical specimens from patients undergoing gastrointestinal surgery. The study was approved by the Norwich District Ethics Committee and carried out in accordance with the Declaration of Helsinki of the World Medical Association. The patients gave informed consent to the work. Samples were immediately cut into portions (~0.1 g) for storage at −70°C until required. On three occasions the tissue samples were prepared and used within 4 h of surgery. Each tissue sample was homogenized in ice-cold phosphate buffer (1 ml), containing 2-mercaptoethanol (10 mM), followed by centrifugation at 13 600×g, 4°C for 30 min. The supernatant was retained and stored on ice prior to use.

2.4. Enzyme assay

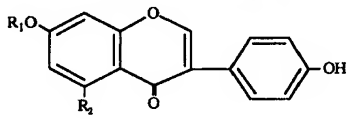
Each flavonoid glycoside was mixed with liver or small intestine extract (containing 200 μg protein) and phosphate buffer, to give a final flavonol concentration of 30 μM in 0.5 ml. Samples were incubated at 37°C for up to 90 min, with controls of heat-inactivated (100°C, 5 min) tissue extract run in parallel. The reaction was stopped by addition of methanol (0.5 ml), containing 0.8 mM ascorbic acid to stabilize the samples during analysis, followed by centrifugation at 13 600×g, for 10 min at 4°C. The supernatant was filtered through 0.22 μm PTFE filter units (HPLC Technology Company, Macclesfield, UK) and analyzed by HPLC.



Flavonoid	R_1	R_2	R_3	R_4
Apigenin 7-glucoside	H	H	H	glucose
Kaempferol 3-glucoside	O-glucose	H	H	H
Q 3-glucoside	O-glucose	OH	H	H
Q 3,4′-diglucoside	O-glucose	OH	glucose	H
Q 4′-glucoside	OH	OH	glucose	H
Q 3-rhamnoglucoside	O-glucose (6←1) rhamnose	OH	H	H



Flavanone	R_1
Naringenin 7-glucoside	glucose
Naringenin 7-rhamnoglucoside	glucose (2←1) rhamnose



Isoflavone	R_1	R_2
Genistein 7-glucoside	glucose	OH
Daidzein 7-glucoside	glucose	H

Fig. 1. Structure of flavonoid and isoflavonoid glycosides.

2.5. Calculation of K_m and V_{max}

Various concentrations of Q 4′-glu and genistin were mixed with aliquots (25 μl) of small intestine or liver extract and phosphate buffer to a final volume of 0.5 ml. All samples were incubated at 37°C for a time period during which the reaction was linear (liver samples, 15 min; small intestine samples, 5 min). The reaction was stopped with methanol/ascorbic acid and treated as described above for HPLC analysis. Protein was determined according to the Bradford method [24], using bovine serum albumin as standard. K_m and the apparent V_{max} were computed using a method described by Wilkinson [25].

The effect of various inhibitors of β-glucosidases was investigated [17]. D-gluconolactone, sodium taurocholate and conduritol B epoxide were incubated at various concentrations with aliquots (50 μl) of liver

Table 1
Initial rates and extent of deglycosylation of (iso)flavonoid glycosides with a small intestine and liver cell-free extract

Substrate	Initial rate of deglycosylation (μmol/min mg)		Deglycosylation after 90 min (%)	
	Small intestine	Liver	Small intestine	Liver
Q 4′-glucoside	2.14±0.20	0.74±0.02	90.0±1.3	68.7±0.1
Q 3-glucoside	0.14±0.03	0	16.2±0.5	0
Q 3-rhamnoglucoside	0	0	0	0
Q 3,4′-diglucoside	0	0	0	0
K 3-glucoside	0	0	0	0
Genistein 7-glucoside	3.15±0.22	1.06±0.22	97.8±0.5	76.3±0.4
Daidzein 7-glucoside	2.40±0.15	0.54±0.06	95.0±0.8	66.5±0.3
Apigenin 7-glucoside	1.35±0.20	0.61±0.13	83.3±0.2	49.0±0.9
Naringenin 7-glucoside	1.05±0.17	0.30±0.09	66.1±2.8	26.3±0.6
Naringenin 7-rhamnoglucoside	0	0	0	0

Samples were incubated for various time intervals (0–90 min) at 37°C. Initial rates were calculated using the linear component of the progress curve only. Alternate time points were assayed in triplicate.

or small intestine extract, glycoside (30 μM) and phosphate buffer to a final volume of 0.5 ml. Each sample was incubated at 37°C for 15 min. The reaction was stopped with methanol/ascorbic acid and treated as described above for HPLC analysis.

2.6. HPLC analysis

A modified version of the analytical HPLC method of Price et al. [26] was used. Solvents A (water:tetrahydrofuran:trifluoroacetic acid, 98:2:0.1) and B (acetonitrile), were run at a flow rate of 1 ml/min, using a gradient of 17% B (2 min), increasing to 25% B (5 min), 35% B (8 min), 50% B (5 min) and then to 100% B (5 min). A column clean-up stage maintained B at 100% (5 min) followed by a re-equilibration at 17% B (15 min). The column was packed with Prodigy 5 μm ODS3 reverse-phase silica, 250 mm by 4.6 mm id (Phenomenex, Macclesfield, UK). Diode array detection monitored the effluent at 270 and 370 nm.

3. Results

Fig. 2 shows representative chromatograms from 0, 30 and 90 min incubations of Q 4′-glu with a cell-free small intestine extract, demonstrating the conversion of Q 4′-glu to quercetin by hydrolysis of the β-glucoside bond. Table 1 shows the rate of deglycosylation of various flavonoid glycosides by a cell-free extract from human small intestine and liver containing equivalent total protein. Q 4′-glu, apigenin 7-glucoside, naringenin 7-glucoside, daidzin, and genistin are all hydrolyzed by the hepatic or intestinal enzymes. The rate of deglycosylation in these samples suggests that the small intestine is more active in this hydrolysis than the liver. Q 3-glu, Q 3,4′-glu, K 3-glu, rutin and naringin are not hydrolyzed by the enzymes from the liver extract in a 90 min period, and with the exception of a small quantity of the Q 3-glu, these compounds are not hydrolyzed by the small intestine extract either. A similar pattern of deglycosylation was seen in all liver and small intestine samples analyzed, with no differences between the freshly prepared and frozen samples.

β-Glucosidase activity at various concentrations of Q 4′-glu and genistin was measured in both the small intestine and liver extracts. The K_m and apparent V_{max} of the reactions were calculated for each tissue sample, based on the rate of formation of the aglycone, and the average values are shown in Table 2. The results suggest that the same enzyme, or one with very similar properties, is responsible for catalyzing the reaction in the liver and small intestine, with a K_m of ~32 μM for Q 4′-glu and ~14 μM for genistin. The total amount of the β-glucosidase activity varies between individuals, although it would appear that the rate of reaction is in general faster in the small intestine than in the liver. This would indicate that the intestinal mucosa plays an important role in the deglycosylation of flavonoids during absorption.

To determine which enzyme was responsible for this activity we used three inhibitors of the human cytosolic broad-specificity β-glucosidase or the membrane-bound glucocerebrosidase [17]. These inhibitors have also been used to characterize the differences between pyridoxine glucoside hydrolase and the broad-specificity β-glucosidase [22].

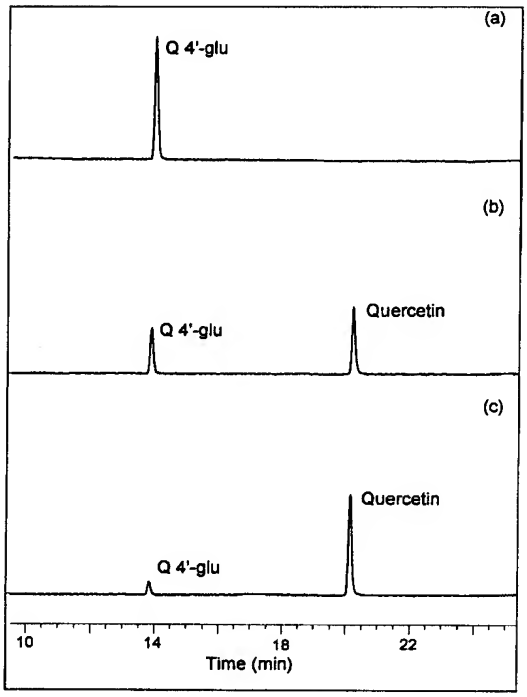


Fig. 2. HPLC chromatogram, monitoring relative absorbance at 370 nm, of Q 4′-glu (30 μM) after incubation with small intestine extract (200 μg protein), at 37°C for (a) 0 min, (b) 30 min, (c) 90 min.

(1) D-gluconolactone is an inhibitor of both the cytosolic and membrane-bound β-glucosidases, although it is much more potent on the cytosolic enzyme at micromolar concentrations.

(2) Sodium taurocholate (1.2%, w/v) enhances the activity of the membrane-bound β-glucosidase, but inhibits both the broad-specificity and pyridoxine glucoside hydrolase. At 0.1% (w/v), sodium taurocholate inhibits the broad-specificity β-glucosidase by 50%, whereas little inhibition of pyridoxine glucoside hydrolase is seen at this concentration.

(3) Conduritol B epoxide (6.2 mM) has little effect on the broad-specificity β-glucosidase, but shows complete inhibition of glucocerebrosidase at this concentration. Conduritol B epoxide inhibits pyridoxine glucoside hydrolase by 70% at 5 mM.

In our experiments, both D-gluconolactone and sodium taurocholate exhibited an inhibitory effect on the β-glucosid-

Table 2
K_m and apparent V_{max} of Q 4′-glucoside and genistein 7-glucoside by cytosolic β-glucosidase from human small intestine and liver cell-free extracts

Substrate		Liver	Small intestine
Q 4′-glucoside	K_m (μM)	27 ± 13 (n = 7)	37 ± 12 (n = 4)
	V_{max} (mU/mg protein)	0.35–1.38	1.06–1.51
Genistein 7-glucoside	K_m (μM)	13 ± 1 (n = 3)	14 ± 5 (n = 3)
	V_{max} (mU/mg protein)	1.03–1.34	1.50–2.91

One unit of activity is defined as one μmol of product formed per min at 37°C. K_m values are expressed as mean ± standard deviation, whereas V_{max} is shown as the range of values measured.

Table 3
Effect of various inhibitors on β-glucosidase activity

β-Glucosidase	Sodium taurocholate (1.2%, w/v)	Gluconolactone (10 mM)	Conduritol B epoxide (5 mM)
	% of control activity		
Glucocerebrosidase [17]	862	49	0[c]
Broad-specificity [17]	16	7	100[c]
Pyridoxine glucoside hydrolase [22]	79[a]	0[b]	30
Human liver cell-free extract	2	0	95
Human small intestine cell-free extract	10	0	83

[a]1%, w/v; [b]based on $K_i = 7$ μM; [c]6.2 mM.

ase activity as shown in Table 3. In addition, sodium taurocholate caused 60% inhibition at 0.2% (w/v). The β-glucosidase activities of the small intestine and liver extracts were inhibited by ~5 and 17% respectively by conduritol B epoxide (5 mM). The effects of these inhibitors are in general agreement with Daniels et al. [17] and suggests that the β-glucosidase reaction is carried out by the cytosolic broad-specificity β-glucosidase.

4. Discussion

This study shows that human small intestine and liver have a β-glucosidase capable of efficiently hydrolyzing various naturally occurring flavonoid and isoflavonoid glycosides. This enzyme has a relatively high affinity for Q 4′-glu as a substrate. The small intestine β-glucosidase was also capable of slowly hydrolyzing Q 3-glu, although the liver was not. Both the hepatic and intestinal β-glucosidases were not active on the exolytic rhamnose of rutin and more surprisingly, the 4′-glucose from Q 3,4′-glu. Therefore, either Q 3-glu and Q 3,4′-glu do not fit into the active site of the enzymes, or they bind to form a non-productive complex. The small intestine may have an additional enzyme which has a low activity towards Q 3-glu, although whether this reaction is of physiological importance, due to the short transition time in the intestinal mucosa, is questionable.

Dietary flavonols are more frequently found with a sugar attached in the 3 position, compared to the 7 and 4′ positions. Other types of flavonoids, which are devoid of a hydroxyl group at position 3, are glycosylated mainly in the 7 position. We therefore investigated the specificity of the β-glucosidase activity from the small intestine and liver for other naturally occurring 7-glycosylated flavonoids. The isoflavone, flavone and flavanone 7-glucosides were all substrates for the β-glucosidase, in contrast to naringin, a 7-rhamnoglucoside. The β-glucosidase had a higher apparent affinity towards genistein 7-glucoside than quercetin 4′-glucoside. Table 4 shows the published K_m values for various β-glycoside substrates with liver cytosolic β-glucosidase. The β-glucosidase in human liver has a higher affinity for the flavonoid and isoflavonoid glycosides than for the other dietary compounds previously investigated and, therefore, these compounds could be substrates in vivo for this enzyme.

Only three human β-glucosidases have been reported to date, with a fourth enzyme identified in porcine intestinal mucosa. We investigated the potential for the cytosolic broad-specificity β-glucosidase to be the enzyme responsible for the activity observed in the human tissue extracts, by studying the known inhibitors or activators of the cytosolic β-glucosidase and the glucocerebrosidase. The results are in agreement with Daniels et al. [17], which indicate that the activity is due to the cytosolic β-glucosidase, not a membrane-bound enzyme. Pyridoxine glucoside hydrolase exhibits a different pattern of inhibition with conduritol B epoxide and sodium taurocholate to the broad-specificity β-glucosidase, and to the enzyme active on the flavonoid glycosides in our tissue samples. It would, therefore, seem likely that the activity demonstrated by the human small intestine and liver extracts results from the cytosolic broad-specificity β-glucosidase.

The deglycosylation of (iso)flavonoids by human cytosolic β-glucosidase could be an important first step in their uptake, metabolism, excretion and biological activity, which is independent of metabolism by the colonic microflora. The results emphasize that many dietary (iso)flavonoid glycosides are deglycosylated by enzymes in human tissues. However, the rate and extent of deglycosylation depends on the structure of the flavonoid and the position/nature of the sugar substitutions. The results are important to consider in any future studies of human metabolism of flavonoid glycosides in vivo.

Acknowledgements: We would like to thank the Biotechnology and Biological Sciences Research Council for funding and for a Ph.D. studentship (to A.J.D.).

Table 4
K_m of various purified liver β-glucosidase substrates

Substrate	Enzyme source	K_m (mM)	Reference
4-Methylumbelliferyl-β-glucoside	human	0.062	[17]
p-Nitrophenyl glucoside	rabbit	2.1	[16]
17 α-estradiol-3-glucoside	rabbit	5×10^{-7}	[16]
17 α-estradiol-17-glucoside	rabbit	0.039	[16]
17 β-estradiol-17-glucoside	rabbit	1.3	[16]
Pyridoxine 5′-glucoside	human	2	[21]
L-picein	guinea pig	0.63	[18]
Q 4′-glucoside	human (crude extract)	0.027	–
Genistein 7-glucoside	human (crude extract)	0.013	–

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